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Research Article

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Demonstration of Macromolecular Inhibitor(s) of Calcification and Nucleational Factor(s) in Fluid from Calcifying Sites in Cartilage

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ABSTRACT An extracellular fluid phase (C_{fl}), aspirated by micropuncture techniques from the hypertrophic cell zone of calcifying epiphyseal cartilage, has been characterized in a calcifying system in vitro in respect to the behavior of sedimenting and supernatant fractions after high speed ultracentrifugation. To perform these tests on the starting samples of 20 nl of C_{fl}, macroscopic analytical methods were scaled down for the identification of relevant organic components, including hexuronic acid and proteinpolysaccharides (PPL). The mineral accretion system was designed to simulate physiologic conditions in the calcifying cartilage septa of normal rats, and the mineral used for seeding was an immature calcium phosphate similar to native cartilage mineral. Normal C_{fl} or its dilutions in synthetic lymph up to 1:4 completely prevented mineral accretion in vitro. The inhibitory action was localized to the sedimented fractions after ultracentrifugation and could be destroyed by incubation with trypsin or hyaluronidase. The sediment of C_{fl} contained 2 mg of hexuronic acid per ml of C_{fl} and gave a strong reaction of identification for a light fraction of PPL by fluorescent antibodies to rat PPL. PPL fractions were tested in the same mineral accretion systems as C_{fl} and exhibited responses similar to those of C_{fl}. Also, there was evidence of a mineral phase in C_{fl} of normal rats, in C_{fl} of rats with healing rickets, but not in C_{fl} of untreated rachitic rats. These results are interpreted to indicate that certain PPLs function as an inhibitor of crystal growth at extracellular sites premonitory to calcification. Evidence for a low density inhibitor of mineral accretion was found in normal serum but not in C_{fl}.

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INTRODUCTION

Collection of a microscopic extracellular fluid phase from the distal hypertrophic cell zone of upper tibial endochondral plates in vivo was demonstrated in a previous report (1). This sampling technique has rendered possible some new direct measurements involving physiological parameters of calcification (1). In the initial study of this calcifying cartilage fluid (C_{fl}), a profile of electrolytes was compatible only with an extracellular origin of the samples (1). Also, an elevated bicarbonate and pH in C_{fl} from normal rats and a high level of inorganic phosphate in C_{fl} from rachitic rats in relation to serum were found (1). Histological evidence from injection of markers into the cartilage, after C_{fl} collection, indicated that this C_{fl} arises predominantly from septal or lacunar extracellular spaces at sites premonitory to calcification (1).

In further studies, partition of normal C_{fl} by ultracentrifugation indicated a surprisingly large component of acid-soluble phosphate in the sediment fraction (1). The fact that this sedimenting phosphate was absent from C_{fl} of (noncalcifying) rachitic cartilage made clear the probability of a mineral phase in normal C_{fl}. However, to explain the demonstrated stability of calcium and phosphate partition in normal C_{fl} for prolonged periods in vitro (1), inhibitors of mineral particle growth in C_{fl} would be required. In this regard, inhibitors of in vivo systems of calcification have been identified, e.g., pyrophosphate by Fleisch and Neuman (2), acidic peptides by Howard, Thomas, Barker, Smith, and Wadkins (3), and certain proteinpolysaccharides by DiSalvo and Schubert (4).

A direct way to test for such inhibitors in C_{fl} is the addition of C_{fl} fractions to an in vitro calcifying system

in which crystals or amorphous particles of calcium phosphates are exposed to a synthetic lymph with an electrolyte composition like that of normal C_{rl} (1). Mineral particle growth would proceed rapidly by removal of calcium and phosphate ions from solution unless blocked by an inhibitor. Similarly, mineral particles (or much less likely an organic nucleational agent) in C_{rl} should cause spontaneous mineral particle growth in this system, if the inhibitors could be inactivated. The subject of this report is the study of mineral accretion in vitro,[1] as effected by PPLs and C_{rl}, or fractions thereof, from normal, rachitic, and healing rachitic rats with inclusion of controls.

METHODS

Fluid collection and analysis. Female Sprague-Dawley rats, of the Holtzman strain, were randomly assigned at age 3 wk to a regimen of normal Purina laboratory chow or USP rachitogenic diet No. 12, as previously described (1). After 23–25 days on this regimen, some of the animals received a massive oral dose of vitamin D_2 (Drisdol) 2000 IU, together with a normal diet for 24 hr. The control animals were continued on normal or rachitogenic diets. All animals on the 24–26th day of the experiment received intraperitoneal injections of Nembutal and underwent collection of 20–40 nl of clear C_{rl} from the hypertrophic cell zone or from the perichondrial and resting cell zone of the tibial epiphyseal cartilage (L_{rl}) as was previously described (1). Immediately thereafter, blood was drawn from the aorta and the animals were sacrificed for histologic study of the cartilages. Methods for animal preparation, micropuncture of the cartilage plates, identification of the fluid collection site, ultracentrifugation of the microscopic fluid samples, with separation of supernatant[2] from sediment fractions, and measurement of calcium, phosphate and pH in these microscopic fractions have been previously reported in detail (1). Total calcium was measured by the method of Clark and Collip (5) and total acid-soluble inorganic phosphate (P_i) by the method of Kuttner and Cohen (6) as modified by Polley (7), in some macroscale experiments (Fig. 1, solid bars). Otherwise, previously described microscale methods for calcium and phosphate were used (1).

A new method, developed for the current study, was a scaled-down modification for hexuronic acid determination as described by Dische (8). 20 nl of C_{rl} was diluted in 2.0 μl of distilled water to which was added 18 μl of 36 N H_2SO_4. The mixture was sealed in a capillary tube and incubated at 100°C for 20 min. After cooling to room temperature, the mixture was subjected to reverse centrifugations four times and one end of the tube opened. 0.5 μl of 0.1% alcoholic solution of carbazole was added with a micropipet. The tube was resealed, thermally, and reverse centrifugations applied four more times. Solutions were then incubated at room temperature for 2 hr and transferred to the ultramicrocuvette. The absorbance was determined at 530 mμ in

[1] Mineral accretion is used here to mean growth of a mineral particle fraction without implications in respect to an increase in size of mineral particles versus number of new particles.

[2] The *supernatant* fraction is defined as the fluid phase comprising the top one-fifth of the column of fluid in the micropipet and because of the sloping pipet walls includes about one-half the total volume of fluid sample. The *sedimented* or *macromolecular* fraction is the packed solid phase in the pipet tip not dislodged by reverse centrifugation at 20,000 *g* at 5°C for 10 min.

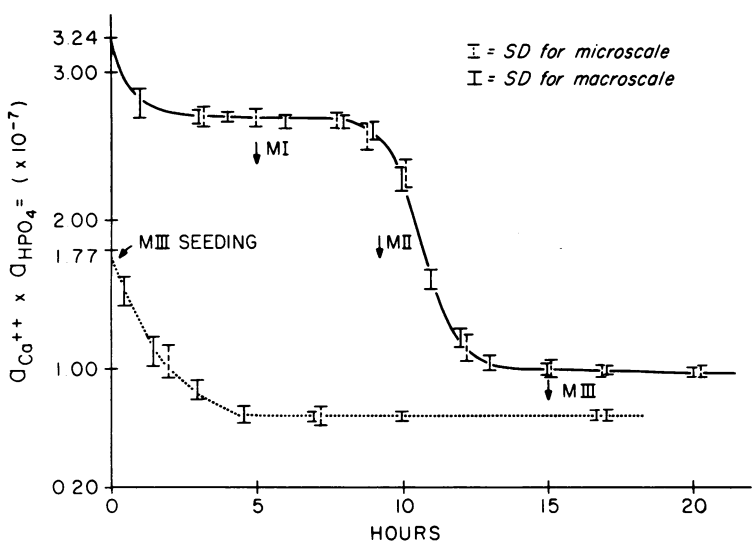

FIGURE 1 Comparison of macroscale (solid bars) and microscale (dashed bars) measurements of calcium and phosphate in a system undergoing spontaneous precipitation (solid line) and a system of crystal growth seeded with MIII calcium phosphates (dotted line). MI, MII, and MIII signify calcium phosphates separated by centrifugation at 5, 10, and 15 hr after onset of precipitation. Each bar represents SD for six to eight experiments.

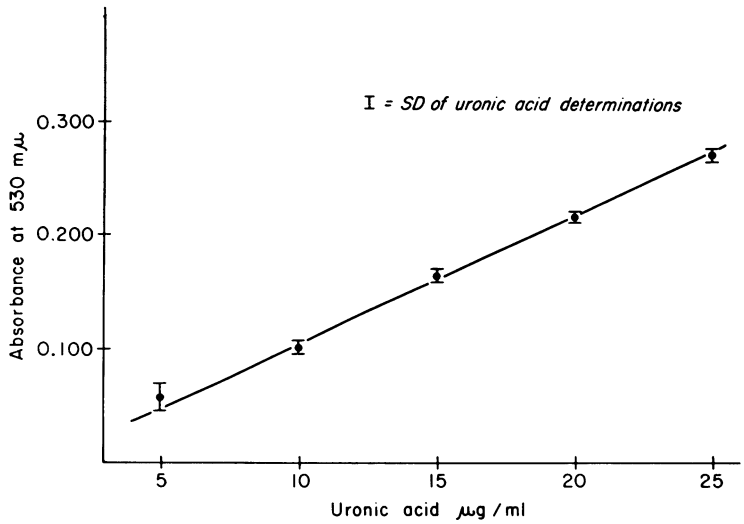

FIGURE 2 Ultramicroanalysis of hexuronic acid by a modified method of Dische. Demonstration that Beer's law is obeyed by standards of uronic acid in the range of 10^{-6}–10^{-10} g.

the Zeiss PMQ II Spectrophotometer (Fig. 2). Recovery values for the hexuronic acid in crude PPL added to C_{f1}, 20 nl samples, were 93–109% (n = 6) at the 10 mg/ml level and 95–105% (n = 6) at the level of 20 mg/ml.

Preparation of mineral phases used for seeding. In an effort to duplicate physiological conditions within the cartilage, several mineral phases were prepared in a synthetic lymph resembling in its constitution C_{f1} as previously reported (1). Mineral phases from various epiphyseal cartilages contain magnesium, as well as carbonate, (9) and have been shown to provide X-ray diffraction powder diagrams interpreted to indicate amorphous calcium phosphates (10). Inasmuch as the precise nature of the mineral phase in cartilage is unknown, calcium phosphates were synthesized under conditions which were varied with respect to pH, duration of mineral maturation, and Ca to P ratio (Table I). Although several fractions of calcium phosphates were separated, after spontaneous precipitation at time periods varying from 5 to 15 hr after mixing, study was made of only two fractions precipitated in fluid at pH 7.4 (MII, MIII, Table I) and one at pH 7.6 (MIV, Table I); these mineral phases were selected on the basis of rapid seeding capacity and sufficient stability for manipulation. The starting activity product $\alpha Ca^{++} \times \alpha HPO_4^{=}$ was approximately 3.2×10^{-7} in the precipitating solution used for production of these minerals.[3]

TABLE I

Results of X-Ray Diffraction Analysis on Mineral Fractions Used for Seeding Experiments

Mineral phase	Duration of precipitation	pH of precipitation	Ca:P molar ratio	Pattern identification
	hr			
MII	10	7.4	1.0 ±0.1*	Amorphous
MIII	17	7.4	1.3 ±0.05	Apatite, poorly crystallized
MIV	3	7.6	1.1 ±0.1	Amorphous

* In this and subsequent tables, data are expressed as mean ± SD.

Spontaneous precipitation of calcium phosphates was carried out with equal volumes (2.5 ml) of two stock solutions of the following composition: (A) 4.00×10^{-3} M $CaCl_2$; (B) 2.50×10^{-1} M NaCl; 6.20×10^{-3} M KCl; 1.10×10^{-3} M $MgCl_2$; 4.60×10^{-2} M $NaHCO_3$; 1.54×10^{-3} M NaH_2PO_4; 2.28×10^{-3}M Na_2HPO_4. Next, CO_2 was bubbled through solution (B) under oil until the pH was 6.8–7.0. Solutions (A) and (B) were then mixed and the pH allowed to increase rapidly to 7.4 or 7.6 at zero time. Precipitation commenced within the incubation mixtures which were kept agitated at 37°C under 5% CO_2-equilibrated oil in sealed tubes for periods which in separate experiments varied from 0.5 to 21 hr. Final ionic strength was approximately 0.16. At each of the times specified in Fig. 1, mineral phase maturation was interrupted by rapid cooling to 5°C and the solids separated by ultracentrifugation of the total sample at 30,000 *g* for 30 min at 5°C in a Spinco model L ultracentrifuge.[4] Ca and P_i were measured on supernatant and sediment fractions and final pH with a Beckman model 76 pH meter. The computed $\alpha Ca^{++} \times \alpha HPO_4^{=}$, remaining in the solution as a function of time at pH 7.4, is shown by the upper curve, solid bars in Fig. 1. There was an initial rapid fall in concentrations of the ions, followed by a lag period attributed to Oswalt ripening (11), then a steep slope during which there was presumably hydrolysis of early embryos to crystalline apatites (11–13), and finally, a phase

[3] Reference to the activity product $\alpha Ca^{++} \times \alpha HPO_4^{=}$ is made in this report to facilitate comparison of experimental conditions with those of previous studies (1, 2, 12). Corrections for calcium associated with bicarbonate and other microanions are not included.

[4] At 5, 10, 15 hr (upper curves, Fig. 1) wherein solids were separated by ultracentrifugation, solids were also separated from 18 additional samples by passage through Millipore filters (pore size 0.2 μ) at 20°C; Ca and P_i measured on sediments separated in this manner provided mean values identical or closely similar with those obtained by ultracentrifugation.

considered to represent slow maturation of mineral crystals (Fig. 1).

Characterization of the seeding mineral phases. The mineral phases which were separated at 5, 10, and 15 hr of incubation at pH 7.4 and after 5 hr of incubation at pH 7.6 were characterized by Ca and P analysis on wet-ashed samples of lyophilized mineral phases, as well as micro X-ray diffraction studies (Table I). For all samples, 1–2 mg of lyophilized mineral powder was packed into capillary tubes and for X-ray diffraction analysis, nickel-filtered copper K-alpha radiation ($\lambda = 1.54050$ A) from a Jarrell-Ash microfocus unit, and a 114.59 mm diameter Debye-Sherrer camera were used. The mineral phases MII and MIV, with a Ca to P molar ratio of 1.0, provided broad peaks in the 2.80 A and 3.44 A reflections which were interpreted to indicate amorphous calcium phosphates (Table I). MIII mineral phase showed peaks at these reflections indicative of poorly crystallized apatite, and the Ca to P molar ratio was 1.3 (Table I). The very low Ca to P ratio of these mineral phases is similar to that reported by Fleisch and Neuman (2) and Boulet and Marier (13), when precipitation was carried out with a solution containing a starting $\alpha Ca^{++} \times \alpha HPO_4^{=}$ only slightly above the solubility product constant (K_{sp}) of brushite. Moreover, the low Ca to P ratio of these salts is considered likely to represent the ratio in a mineral phase postulated (from data presented later in this report) to form in C_{f1} in vitro. Also, there was a low ratio of Ca to P in the macromolecular fraction of C_{f1} (1) and in mineral-containing fractions of epiphyseal hypertrophic cell zone, after correction for acid-insoluble P (14).

Experimental system for the study of crystal growth and the miniaturization of this system. In the macroscale system for mineral accretion, 2.5 ml each of solutions (A) and (B), described above, were mixed under oil, equilibrated with 5% CO_2 in the presence of MIII mineral powder, and 0.2 mg/ml added initially to the reaction vessel. In some experiments PPL and enzymes were mixed into solution (B) with a Disontegrator.[5] The starting concentrations of Ca and P_i in the mixed solutions, were adjusted so that computed initial $\alpha Ca^{++} \times \alpha HPO_4^{=} = 1.77 \times 10^{-7}$. As in the spontaneous precipitation system for preparing mineral phases (Fig. 1), the fluid was incubated in sealed tubes with agitation for periods ranging from 0.5 to 48 hr. Mineral maturation was interrupted by chilling the reaction mixture to 5°C and ultracentrifugation for 30 min at 30,000 *g* and 5°C. All supernatant samples were analyzed for Ca and P_i. With every fifth sample, Ca and P_i were analyzed upon the sediment fractions as a check on the methodology. Good agreement between the latter values and loss from supernatant fractions was obtained.

Next, this entire procedure was miniaturized and the kinetic curves of computed $\alpha Ca^{++} \times \alpha HPO_4^{=}$ in a system with a total reaction mixture of 100 nl were compared to the results of the macroscale measurements (Fig. 1). The microscale mineral seeding studies were performed in an identical manner with those on a macroscale, except that 50 nl each of solutions (A) and (B) were mixed under CO_2-equilibrated oil which was sealed in a siliconized concavity slide.[6] After incubation at 37°C for appropriate periods, the samples were aspirated into micropipets, the tips sealed, and after ultracentrifugation, the supernatant fractions were delivered under oil. Supernatant fractions were then analyzed for Ca, P_i, and pH. The mineral accretion experiments began with additions to the sealed concavity slide of solutions (A) and (B) under CO_2-equilibrated oil. In some experiments, either supernatant fraction of C_{f1} or total C_{f1} was substituted for synthetic lymph. In other experiments, sediment fractions of C_{f1} were added 1:1 to synthetic lymph, as described above, with or without enzymes. With the use of this technique, it can be seen that disappearance of Ca and P_i from the supernatant fraction of seeded solutions was essentially the same in both micro- and macroscales (lower curve, Fig. 1). The steep slope of disappearance from supernatants of control solutions, seeded with MIII mineral, ended at 4½ hr. Thereafter, there was no detectable loss of these ions or only a slight decline. For this reason, presence or absence of inhibition of mineral accretion was determined by simply the 0 and 5½ hr observations, in most experiments, after seeding with MIII minerals. Reliability of the microscale measurements was substantiated by the results of spontaneous precipitation experiments (upper curve, dashed bars, Fig. 1), which indicated close conformity to macroscale results.

Preparation of proteinpolysaccharides, fluorescent antibodies, and enzymes. The proteinpolysaccharide light fraction 5 (PPL 5) was prepared as described in detail by Pal, Doganges, and Schubert (15). The PPL 5, used for our studies, revealed the following characterization: absorbance of 1% solution at 280 mμ, 2.50; 27% protein; 19.9% hexuronic acid; 23.2% hexosamine; 16.5% galactosamine; 1.2% sialate; 0.18% hydroxyproline; and chain weight $\times 10^{-3}$, 109.0. Sedimentation of the PPL 5 fraction, performed in a Spinco ultracentrifuge at 56,100 rpm from a solution of 0.05 M phosphate, pH 7.0, and 0.10 M KCl at 20°C revealed a single sharp peak (15). For control experiments (Fig. 6), PPL 3 was prepared from bovine nasal cartilage; the starting whole PPL contained 23.4% protein, 21.2% hexuronic acid, 20.8% hexosamine, and 3.5% hexoses (15). For some experiments (Table V, Fig. 5), a mixture of chondroitin sulfate A and C was employed; this preparation, isolated from bovine cartilage, was contaminated with < 5 g of protein per 100 g dry weight. Purified testicular hyaluronidase, 850 TRU/mg,[7] and purified trypsin[7] (twice recrystallized by the authors) were employed for other experiments (Tables III, IV, Figs. 3 and 5).

Unlabeled and fluorescein isothiocyanate–labeled antibodies to rat bovine whole PPL (see Acknowledgments) were prepared according to the methods referred to and modified in the report of Hirschman and Dziewiatkowski (16).

Application of the fluorescent antibody technique to identification of proteinpolysaccharide in C_{f1}. We then devised a method to fix the residue of 20 nl of C_{f1} in spots 100–200 μ in diameter upon the glass slides used for fluorescence microscopy. The conventional washing procedure, required for removal of excess labeled antibody, solubilized the spots when different types of gelatin fixation were attempted. The MIII mineral phase not only adhered tightly to the glass slides after preliminary drying, but bound the C_{f1} residue, as well as control proteinpolysaccharide and chondroitin sulfate preparations. Autofluorescence was absent in the MIII mineral phase. C_{f1} samples were then mixed with one-tenth the volume of MIII mineral lyophilized powder and fixed to the slides with absolute alcohol. PPL, chondroitin sulfate, and rat serum globulin were carried through the same procedure in which test spots of these materials were overlaid with either fluorescent or nonfluorescent antibodies inside a sealed humid chamber at 20°C for 1 hr. The spots were next washed with buffered saline repeatedly for 15 min,

[5] Ultrasonic Industries, Inc., Plainview, N. Y.

[6] Clay-Adams A-1475, Clay-Adams, Inc., New York.

[7] Worthington Biochemical Corp., Freehold, N. J.

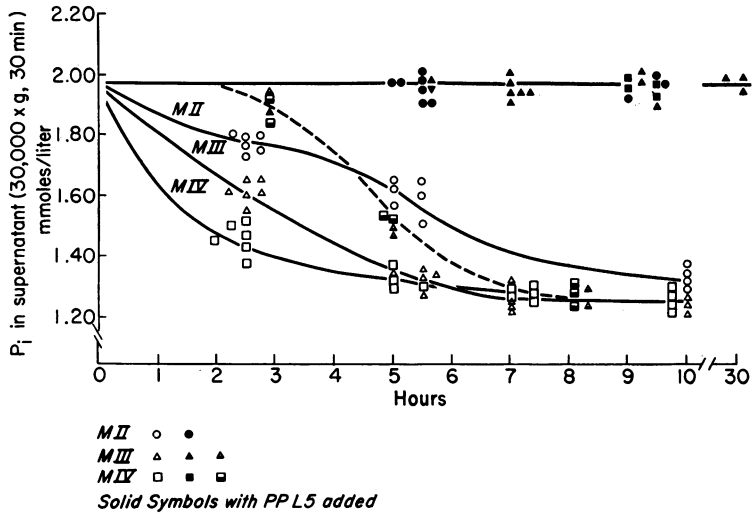

FIGURE 3 Demonstration of complete inhibitory action of proteinpolysaccharide (PPL 5) (1 mg/ml) (solid symbols) upon mineral accretion with three seeding calcium phosphate preparations, (MII, MIII, MIV) when 0.2 mg/ml of the mineral preparations was added to synthetic lymph at time zero. Inhibition continued for at least 30 hr after MIII seeding. Control curves of mineral accretion are shown by open symbols. Treatment with hyaluronidase 750 TRU/ml, resulted in destruction of PPL 5 inhibitor (dashed line, semisolid symbols).

and the slides were mounted in equal parts of glycerine and buffered saline. Examination of the spots was made with a Leitz Wetzler (Ernst Leitz Ltd., Midland, Ontario) fluorescence microscopy assembly with a mercury arc lamp CS 150 w, heat filter, KG I, Blue filter, BG 12, and yellow barrier filter. Photographs were taken with a Polaroid camera at 125× magnification with 3000 speed type 107 film, employing 5-min exposures.

RESULTS

Data gathered on the behavior of proteinpolysaccharide in the vitro model system of mineral accretion on a macroscale (Figs. 3–5) served as both a methodological control and physiological basis for comparison of results obtained on cartilage fluids studied on an ultramicroscale (Tables II–V, Figs. 6 and 7).

Behavior of C_{fi} in the mineral accretion system. Screening experiments were performed to determine the effect of fresh unaltered C_{fi} of normal and healing rachitic rats on microscale mineral accretion (Table II). The Ca and P_i of control synthetic lymph decreased by 0.68 and 0.92 mmoles/liter at $5\frac{1}{2}$ hr after mixing; in contrast, C_{fi} of normal and healing rachitic animals, added to the system, prevented significant alterations in the fluid concentration of these ions (i.e., total inhibition) after the same standard incubation period of $5\frac{1}{2}$ hr.

It was observed that the inhibitory activity on mineral accretion, demonstrated first in whole C_{fi}, was limited to the sedimenting fractions of C_{fi} samples (Table II). It was next important to assess the potency of this inhibitory action of C_{fi} on mineral accretion in terms of the highest dilution effective (Fig. 6). It can be seen that dilutions of 1:1 and 1:4 of C_{fi} in synthetic lymph

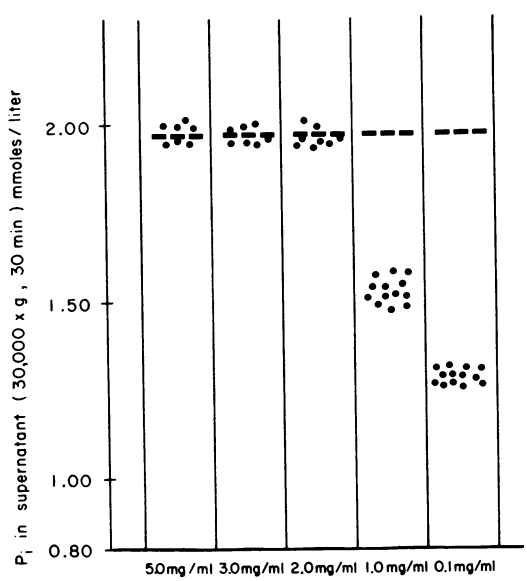

FIGURE 4 Effect of varying the concentration of PPL in synthetic lymph on inhibition of mineral accretion seeded with MIII mineral. Mean concentration of inorganic phosphate (P_i) in solution is shown before incubation (dashed line) and separate concentrations (solid dots) for individual rat samples after 7 hr of incubation at 37°C and pH 7.6.

TABLE II

The Inhibitory Effect on Mineral Accretion of Serum and Cartilage Fluid (C_{fl}) from Normal and Healing Rachitic Rats

Experimental conditions*		No. of samples	ΔCa‡	ΔPi‡	ΔCa:ΔPi	Inhibitory action
			mmoles/liter	*mmoles/liter*		
Synthetic lymph		20	−0.92 ±0.04	−0.68 ±0.02		—
Normal serum	Whole	14	+0.05 ±0.07	−0.08 ±0.04		Positive
	Supernatant	16	0.00 ±0.08	+0.03 ±0.05		Positive
Normal C_{fl}	Whole	16	−0.02 ±0.05	−0.04 ±0.03		Positive
	Supernatant	24	−0.87 ±0.03	−0.70 ±0.05	1.24	Negative
	sediment	6	+0.08 ±0.05	0.00 ±0.02		Positive
Healing rachitic serum	Whole	8	+0.02 ±0.06	−0.07 ±0.08		Positive
	Supernatant	6	+0.03 ±0.08	−0.03 ±0.06		Positive
Healing rachitic C_{fl}	Whole	8	+0.03 ±0.06	0.00 ±0.06		Positive
	Supernatant	8	−0.89 ±0.07	−0.78 ±0.05	1.14	Negative

* Approximately 20 nl of all indicated specimens were incubated under oil for 5½ hr at 37°C and pH 7.60 after addition of MIII (0.2 mg/ml). "Whole" indicates untreated samples. Supernatants and the normal C_{fl} sediments were obtained after centrifugation of samples at 130,000 *g* for 8 hr at 5°C. The sediments were resuspended in a volume of synthetic lymph equivalent to starting C_{fl}.

‡ Calcium and inorganic acid-soluble phosphate (P_i) remaining in solution after centrifugation of the samples at 30,000 *g* for 30 min; 5°C were substracted from the values at time zero.

with starting $\alpha Ca^{++} \times \alpha HPO_4^{=} = 1.77 \times 10^{-7}$ did not deter effective inhibition, but that in a dilution of 1:10, C_{fl} was unable to prevent mineral phase separation in the presence of MIII mineral. By way of further characterization, whole C_{fl} of normal and healing rachitic rats, treated with trypsin or testicular hyaluronidase, caused total loss of inhibitory function of the C_{fl} on mineral accretion (Table III). Specific actions of these enzymes were indicated inasmuch as heating abolished their effect (Table III). Also, macromolecular inhibitory

TABLE III

Removal of Inhibitory Properties of C_{fl} in Respect to Mineral Accretion by Incubation with Trypsin or Hyaluronidase after Seeding with MIII

Composition of incubated systems*	No. of samples	ΔCa	ΔPi	ΔCa:ΔPi	Inhibitory action
		mmoles/liter	*mmoles/liter*		
1. Synthetic lymph	6	+0.01 ±0.05	0.00 ±0.02	—	—
2. Normal C_{fl}; MIII; inactivated hyaluronidase; synthetic lymph	6	−0.05 ±0.07	−0.06 ±0.05	—	Positive
3. Normal C_{fl}; MIII; hyaluronidase; synthetic lymph	10	−1.16 ±0.04	−0.86 ±0.04	1.35	Negative
4. Normal C_{fl}; MIII; inactivated trypsin; synthetic lymph	6	+0.05 ±0.06	−0.03 ±0.04		Positive
5. Normal C_{fl}; MIII; trypsin; synthetic lymph	8	−1.09 ±0.07	−0.87 ±0.02	1.25	Negative
6. Same as 3. except healing rachitic C_{fl}	6	−1.22 ±0.06	−0.92 ±0.03	1.33	Negative
7. Same as 5. except healing rachitic C_{fl}	6	−1.22 ±0.08	−0.92 ±0.03	1.33	Negative
8. Same as 5. except normal serum	6	0.00 ±0.05	+0.01 ±0.06	—	Positive

For abbreviations see Table II.

* Experimental conditions are described in Table II, except for dilution of samples 1:1 with synthetic lymph containing: 0.4 mg/ml of MIII and 2 mg/ml of trypsin or 1500 TRU/ml of testicular hyaluronidase.

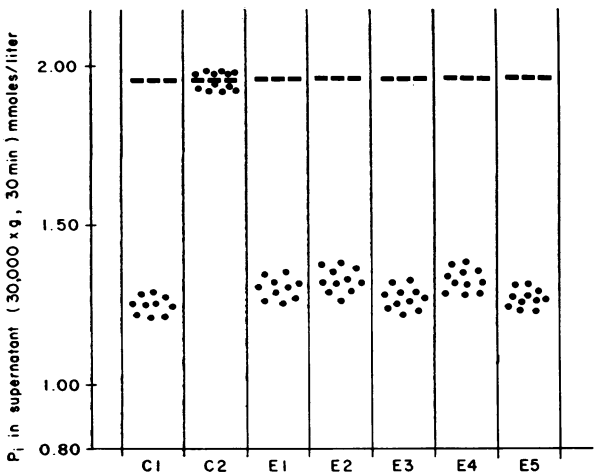

FIGURE 5 Separate effects of degradative enzymes and dilution during 7 hr incubation upon PPL 5 inhibition of mineral accretion with preparations of premixed PPL 5, and MIII mineral, 0.2 mg/ml. Failure of inhibition with PPL 3 and chondroitin sulfate are also shown. Mean concentration of P_i in solution before incubation (dashed line) and separate concentrations (solid dots) after 7 hr of incubation 37°C and pH 7.6. In addition to mineral, starting constituents were: (C 1), synthetic lymph alone; (C 2), synthetic lymph, PPL 5, 1 mg/ml, and inactivated trypsin 1 mg/ml; (E 1), synthetic lymph, PPL 5, 1 mg/ml, and trypsin, 1 mg/ml; (E 2), synthetic lymph, PPL 5, 1 mg/ml, and hyaluronidase, 750 TRU/ml; (E 3), synthetic lymph and PPL 5, 1 mg/ml, further diluted 1:10; (E 4), synthetic lymph and PPL 3, 5 mg/ml; (E 5), synthetic lymph and chondroitin sulfate, 1 mg/ml.

activity of undiluted C_{fl} and PPL 5, 1 mg/ml of synthetic lymph, was destroyed by heating to 70°C for 20 min (n = eight rats for each experiment).

Inhibitory properties of PPL on mineral accretion. The behavior of whole PPL and the PPL 5 fraction was examined in the same mineral accretion system (Figs. 3–5) which was used for C_{fl}. The system was seeded with three separate mineral fractions, which differed most importantly from each other in respect to stage of maturation (MII, MIII) and pH of precipitating solution (MIV, Table I). In the first control experiments (Fig. 3), these synthetic minerals were added alone to the synthetic lymph which had a starting $\alpha Ca^{++} \times \alpha HPO_4^{=}$ of 1.77×10^{-7}. In these experiments (Fig. 3), mineral accretion after seeding with MIII or MIV was rapid. After seeding with MII, mineral accretion occurred at a slower rate, reaching the same point of P_i removal at 9 hr as with MIII at 7 hr. In the presence of PPL 5, 1 mg/ml in the starting solution, slight or no mineral accretion was demonstrable with any of the mineral preparations during the period of observation which was 10 hr for MII and MIV and 30 hr for MIII (Fig. 3). These findings indicate that the inhibitory property of PPL 5 applies to different calcium phosphate preparations, but MIII mineral phase was used for all other seeding experiments reported here, based on its reproducible behavior (Fig. 3).

In a quantitative study on the inhibitory action of whole PPL upon MIII accretion, PPL was effective in preventing mineral accretion in concentrations ranging from 2–5 mg/ml (Fig. 4). At a concentration of 1 mg/ml of PPL, there was partial inhibition, and at 0.1 mg/ml, there was loss of inhibition (Fig. 4). Also a group of experiments was performed to ascertain the response of the mineral accretion system with PPL 5 under conditions similar or identical with those used for C_{fl} (Fig. 5). MIII mineral phase was added to PPL 5, 0.2 mg/ml, in synthetic lymph and mixed by sonication. After incubation at 20°C for 30 min, the material was centrifuged at 700 *g* for 15 min; no mineral sedimentation occurred. The supernatants, from this spinning, were incubated with trypsin, hyaluronidase, and heat-inactivated trypsin for 7 hr at pH 7.6 and 37°C. Whereas controls treated with preheated enzyme carried for 7 hr continued to show total inhibition, mineral accretion proceeded rapidly after incubation of the PPL mineral suspensions in the presence of these active enzyme preparations as well as after dilution (Fig. 5). Furthermore, the inhibitory effect of PPL 5 was at least partially specific, inasmuch as chondroitin sulfate alone or PPL 3 were ineffective (Fig. 5).

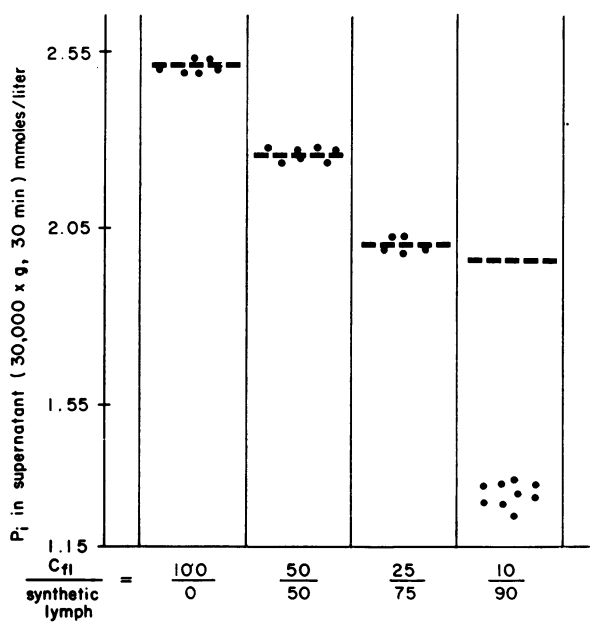

FIGURE 6 Effect of dilution of normal cartilage fluid (C_{fl}) with a synthetic lymph upon the precipitating capacity of MIII mineral phase. Mean concentration at onset of incubation (dashed line) and separate concentrations (solid dots) for individual rat samples at 7 hr of incubation at 37°C and pH 7.6.

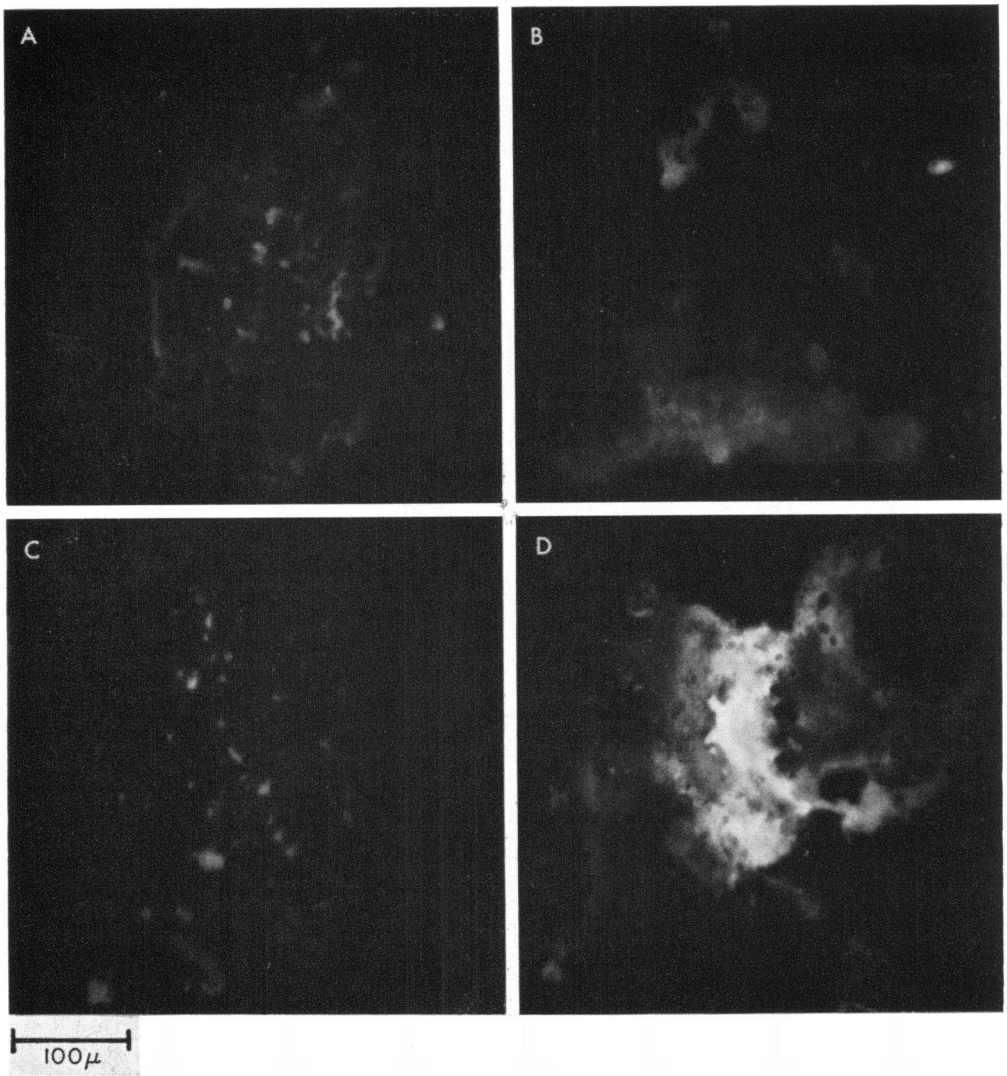

FIGURE 7 Comparison of responses registered in photomicrographs of various residues reacted with fluorescent antibody preparations. Slight or absent fluorescence was shown by: (*A*) MIII (mineral) used in fixation alone and labeled anti-rat PPL antibody; (*B*) MIII, C_{r1} residue, and labeled anti-rat serum globulin; and (*C*) MIII, C_{r1} residue with unlabeled and labeled anti-rat PPL (blocking test). Intense fluorescence was shown by MIII, C_{r1} residue, and labeled anti-rat PPL (*D*). × 160.

Identification of PPL and quantitation of hexuronic acid in C_{r1} sediment. The next aspect of this investigation was an attempt to identify proteinpolysaccharides in C_{r1}. The first of these techniques involved immunological identification of proteinpolysaccharide in whole C_{r1} (Table V, Fig. 7). Residues of C_{r1} from normal rats which had been overlaid with fluorescent antibodies to either rat or calf PPL showed a strong reaction (Fig. 7). In regard to controls (Table V), there was absence of autofluorescence of the MIII mineral phase used to bind reactants to the slide, negative reaction after blocking C_{r1} residues with nonfluorescent anti-PPL antibody, extremely weak reaction for residues overlaid with fluorescent anti-rat globulin, and a negative reaction between anti-PPL and purified chondroitin sulfate. Bovine antibody to rat PPL reacted moderately with C_{r1} residues. Other methodological controls with bovine PPL and anti-bovine PPL indicated that the immunological system applied to microscale samples of antigen reacted satisfactorily with these antibody preparations (Table V). The experimental results demonstrate immunological antigenic components in common between C_{r1} macromolecules and proteinpolysaccharide. From evidence previously discussed concerning specificity of

TABLE IV

Demonstration of a Fraction (s) which Induced Precipitation of Calcium Phosphates in C_{fl} of Normal and Healing Rachitic Rats

Composition of incubated systems*	No. of samples	ΔCa	ΔPi	ΔCa:ΔPi
		mmoles	*mmoles*	
Synthetic lymph; hyaluronidase	8	+0.03 ±0.04	0.00 ±0.02	—
Whole normal L_{fl}‡; synthetic lymph hyaluronidase	6	−0.05 ±0.06	0.01 ±0.04	—
Sediment§ normal C_{fl}; synthetic lymph; inactivated trypsin	8	−0.03 ±0.06	−0.01 ±0.04	—
Sediment§ normal serum; synthetic lymph; hyaluronidase	8	+0.01 ±0.06	−0.02 ±0.04	—
Whole normal C_{fl}; synthetic lymph; hyaluronidase	14	−1.15 ±0.03	−0.85 ±0.04	1.35
Supernatant normal C_{fl} synthetic lymph; hyaluronidase	12	+0.03 ±0.08	0.00 ±0.05	—
Sediment§ normal C_{fl}; synthetic lymph; hyaluronidase	16	−0.90 ±0.04	−0.68 ±0.03	1.32
Sediment§ normal C_{fl}; synthetic lymph; trypsin	6	−0.95 ±0.02	−0.70 ±0.04	1.36
Whole rachitic C_{fl}; synthetic lymph; hyaluronidase	8	0.00 ±0.05	−0.02 ±0.05	—
Sediment§ rachitic C_{fl}; synthetic lymph; hyaluronidase	6	+0.01 ±0.06	0.00 ±0.02	—
Whole healing rachitic C_{fl}; synthetic lymph; hyaluronidase	8	−1.33 ±0.04	−0.98 ±0.03	1.36
Supernatant healing rachitic C_{fl}; synthetic lymph; hyaluronidase	8	+0.06 ±0.05	−0.04 ±0.03	—
Sediment§ healing rachitic C_{fl}; synthetic lymph; hyaluronidase	8	−0.97 ±0.05	−0.71 ±0.05	1.36
Sediment§ healing rachitic C_{fl}; synthetic lymph; trypsin	6	−0.95 ±0.06	−0.75 ±0.05	1.27

For abbreviations see Table II.

Experimental conditions are those described for Table III, except that there was *not seeding with MIII*, and the incubation time was extended to 7 hr.

‡ L_{fl}, represents the fluid aspirated from the peripheral resting cell zone.

§ The sediment was obtained by centrifugation of the samples at 130,000 *g* for 8 hr at 5°C; after removing the supernatant, the material accumulated in the tip was redispersed in the corresponding fluid.

these antibodies (15), the presence of proteinpolysaccharide in C_{fl} is thereby further substantiated.

In the next series of experiments, the hexuronic acid content of normal rat C_{fl} was quantitated and the level of proteinpolysaccharide content estimated, based on the assumption that the hexuronic acid to PPL ratio in bovine nasal cartilages (16) probably applies, roughly at least, to C_{fl} proteinpolysaccharide. Total hexuronic acid was 2.1 ±0.2 mg/ml in normal rat C_{fl} samples (n = six rats), and therefrom 9.5 mg/ml of PPL was computed. Because not all of the measured hexuronic acid is necessarily in the PPL but could be part of free chondroitin sulfate or other macromolecular components, this value must be considered an upper limit.

Demonstration of mineral particles or a nucleational agent for calcium phosphates in C_{fl}. Data suggestive

TABLE V*

Demonstration of Fixation of Fluorescent Anti-PPL Antibodies to Residue of 50–100 nl of Pooled Normal C_{fl} Together with Methodological Controls

Nature of residue tested*	Labeled antibodies to listed antigens‡	Degree of fluorescence		
		0	Slight	Intense
Bovine PPL	Saline alone	6‖	0	0
Rat C_{fl}	Saline alone	2	0	0
MIII mineral used in fixation	Rat whole PPL	4	0	0
Chondroitin sulfate	Rat whole PPL	4	0	0
Bovine PPL	Rat serum globulin	4	0	0
Bovine PPL	Rat whole PPL	2	4	0
Bovine PPL	Bovine PPL 3	0	2	6
Bovine PPL	(*a*) Bovine whole PPL§ (unlabeled)			
	(*b*) Bovine whole PPL	0	4	0
Rat C_{fl}	Rat serum globulin	4	0	0
Rat C_{fl}	(*a*) Rat whole PPL§ (unlabeled)			
	(*b*) Rat whole PPL	4	0	0
Rat C_{fl}	Bovine whole PPL	0	1	4
Rat C_{fl}	Rat whole PPL	0	1	6

* Dried residues fixed with MIII mineral to slides; for PPL samples, a 1.0 g/100 ml solution in 0.8% saline was applied.
‡ 0.5–1.0 mg/ml of protein in buffered saline.
§ Blocking tests.
‖ Number of individual tests.

of calcium phosphate binding in C_{fl} sediments (1), as well as the possibility that such calcium phosphates (Fig. 6) were inhibited by native PPL in C_{fl}, led to a search for a nucleational agent or mineral in native C_{fl}. For this purpose, C_{fl} of normal, rachitic, and healing rachitic rats was incubated for 7 hr with trypsin and hyaluronidase without adding any seeding mineral (Table IV). Mineral accretion occurred spontaneously in the whole C_{fl} and its sedimenting fraction from normal and healing rachitic rats but failed to occur in similar samples from untreated rachitic rats, or in perichondrial fluid (L_{fl}) of normal rats (Table IV). Normal whole serum or serum fractions, sedimenting after ultracentrifugation, contained no mineral or nucleational agent released by enzymes.

The ratio of Ca to P_i removed from the solution at 5½–7 hr of incubation during these and previous experiments in which inhibition was abolished (Table II–IV) ranged from 1.20–1.36.

Evidence for the absence of low density inhibitors of mineral accretion in C_{fl}. A previous study showed that with ultracentrifugation at 100,000 *g* for 8 hr, 5°C, microscopic samples of rat serum contained in the supernatant fraction < 0.8 g of protein per 100 ml (1). Thus the total inhibition of mineral accretion, induced by the supernatant fraction of normal and healing rachitic serum (Table II), is evidence for the presence of one or more low density inhibitory agents in these fluids. Among such agents reasonably expected to fall in this category would be pyrophosphate (2, 17), acidic peptides (3), citrate (18), or magnesium (19). Conversely, total absence of inhibitory activity in normal and healing rachitic supernatant fractions of C_{fl} (Table II), under conditions used to test serum, is evidence against inhibition by these factors of crystal growth in C_{fl}. Failure of trypsin to remove the inhibitory action of serum on mineral accretion further supports the nonidentity of serum and C_{fl} inhibitors (Table III).

DISCUSSION

Similarities of PPL and C_{fl} in the inhibition of mineral accretion in vitro. The likelihood that polysaccharides "shield" reactive sites on collagen fibrils and block mineral nucleation was advanced by Glimcher (20) in 1958. The first direct evidence favoring an inhibitory action of proteinpolysaccharides on calcification was obtained by Weinstein, Sachs, and Schubert who found that sedimentation of calcium phosphates at low centrifugal forces was prevented by these compounds (21). Their observations were extended by DiSalvo and Schubert who noted that proteinpolysaccharides, particularly those contained in a fraction designated in that laboratory as PPL 5, combined stoichiometrically with calcium phosphates (4).

The current data (Fig. 5) not only confirm the results concerning mineral suspension by PPL 5 (4, 21) but, in addition, demonstrate arrest of mineral growth with only slight removal of calcium and phosphate from the solutions in the presence of the PPL 5 fraction (Figs. 3, 5). The conditions in the current experiments differed from those employed by DiSalvo and Schubert (4) in respect, not only to the presence of bicarbonate buffer and magnesium in the incubating lymph, but also to our use of a lower $@Ca^{++} \times @HPO_4^{=}$ of the starting solution. For both of these experimental calcifying systems, intact whole PPL and PPL 5 were effective inhibitors, whereas PPL 3, chondroitin sulfate, or PPL, degraded by trypsin and hyaluronidase, were ineffective. Probably, one or more component proteinpolysaccharides, contained within the whole PPL fraction and enriched in the substituent PPL 5 fraction, are responsible for the inhibitory behavior shown in our mineral accretion system, as well as the capacity to suspend mineral in the aforementioned precipitation system (4). To explain this behavior of whole PPL or PPL 5 on mineral precipitation, DiSalvo and Schubert postulated a weak bonding or mechanical entrapment of calcium phosphates between linear polysaccharide chains, probably at points close to the attachment of polysaccharide to the protein, visualized as a core (4).

Relevance of these observations to calcification in cartilage was indicated by the similarity in effect on mineral accretion by C_{fi} (Table II–IV, Fig. 6) and by PPL or the PPL 5 fraction (Figs. 3–5). The inhibition of mineral accretion by PPL and C_{fi} macromolecular fractions could be abolished by the same methods of enzymatic degradation, dilution, as well as heating. Furthermore, the immunological results of Table V are strongly indicative of PPL in C_{fi}. A hypothetical inhibitory role for PPL in C_{fi} is also compatible with the computed upper limits of PPL content in C_{fi}, based on its total hexuronic acid content in relation to the highest dilution of C_{fi} critical to inhibition (Fig. 6). Thus, if the computed upper limit for PPL in normal C_{fi} (9.5 mg/ml) is the actual value, the concentration of PPL would drop from 2.4 to 1.0 mg/ml upon passing from a dilution of C_{fi} (1:4) totally effective as an inhibitor to one totally ineffective (1:10) (Fig. 6). In the macroscale experiments, the critical level of PPL required for mineral accretion was 2.0 mg/ml (Fig. 4). By exposure to the estimated upper limit of concentration for PPL in C_{fi}, it is possible to account for all the inhibitory activity of C_{fi} on mineral accretion in the experiments of Fig. 4.

Evidence for mineral particles in C_{fi} at calcifying sites. There was evidence of mineral particles, or less likely an organic nucleating agent for calcium phosphates, in the macromolecular fraction of C_{fi} of normal and healing rachitic rats (Table IV). Absence of evidence for mineral particles in C_{fi} of untreated rickets or in the resting cell perichondrial fluid samples of normal rats, as well as in serum, indicates that the findings are not an artifact of microscopic fluid collection and analytic manipulation. At a level of nonprotein-bound Ca and nonprotein-bound P_i previously quantitated in normal C_{fi} (1), spontaneous mineral growth occurred rapidly once the inhibitory component was removed by enzymatic degradation in vitro (Table IV). From these results, it seems likely that during normal rat epiphyseal growth mineral elaboration begins in adjacent hypertrophic cells or possibly the proliferating cell zone with storage of a small mineral phase bonded to proteinpolysaccharides in the cartilage septa. Current data on C_{fi} (Table III, IV) can, in the authors' view, be best explained by the sampling of this stored mineral suspended by and bound to a PPL. These findings are consistent with the concepts discussed elsewhere (22) that the hypertrophic cells adjacent to the calcifying zone degenerate under the influence of invading capillaries and liberate factors destined to degrade PPLs (23, 24) or alter those properties of proteinpolysaccharides which repress mineral growth (25). Thereby rapid mineral phase formation at the zone of provisional calcification could be initiated.

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